Remarks:

The following remarks are provided to further demonstrate the scientific importance of Applicants' discoveries as claimed herein. As commented upon previously (see Applicants' paper mailed December 22, 1992 at pages 5-6 thereof, and Applicants' paper mailed October 3, 1994 at pages 6-7 thereof), substantial difficulties **prevented** the production of stable, recoverable, full length CFTR-encoding DNA. Applicants patent application discloses unobvious solutions to these unexpected difficulties.

To illustrate this, the Examiner's attention is respectfully directed first to Drumm et al. "Correction of the Cystic Fibrosis Defect *in vitro* by Retrovirus-Mediated Gene Transfer" Cell, volume 62, pages 1227-1233. [This reference is of record in the present application by way of Applicants' Information Disclosure Statement]. Two of the co-authors of this article are Drs. Francis S. Collins and James M. Wilson, the named inventors of U.S. Patent 5,240,846, which the Patent Office has cited against the present application. However, the Drumm et al. article, which was **published in September 1990** well **after the March 1990** date to which **the present application** claims priority, acknowledges (at pages 1-2 thereof) that ..."Early attempts to reconstitute a full-length CFTR cDNA from overlapping clones were uniformly unsuccessful." Thus, the difficulties aforementioned were still being experienced well after Applicants' filing date.

It is respectfully submitted that neither any patent application to which U.S. Patent No. 5,240,846 claims priority that was filed before September 18, 1990, nor any publication available before said date, discloses **either the existence of, or the solution to,** the severe difficulties that prevented expression of recombinant cystic fibrosis transmembrane conductance regulator (CFTR) polypeptide - a problem first recognized and solved by Applicants.

The Examiner's attention is further directed to a publication by Dr. Lap-Chee Tsui ("Probing the Basic Defect in Cystic Fibrosis", Current Opinion in Genetics and Development, volume 1, 1991, pages 4-10, **additional copy attached).** This publication was called to the attention of the Patent Office in Applicants' paper of December 22, 1992 (see pages 5-6 thereof) **although it is not prior art.** Dr. Tsui is a coinventor of each of the patent applications (07/396,894; 07/399,945; and 07/401,609) filed in 1989 to which the cited U.S. patent to Collins et al. (No. 5,240,846) claims priority. The Examiner is invited to consider the teaching of the Tsui publication, at pages 7-8 thereof, under "Construction of a full-length CFTR cDNA" wherein Dr. Tsui finally (after applicants' filing date) acknowledges (1) the existence of very substantial cloning difficulties and (2) the presentations of solutions thereto including those by Applicants. **Applicants were cited as among those believed to have first solved the problem .**

Applicants submit that the teachings which provide successful resolution of these difficulties, and therefore for the first time **enable** production of a CFTR-encoding DNA that may be cloned and recovered in bacteria, are first provided in U.S. patent application 07/488,307, filed on March 5, 1990, which is a parent of the present application.

Please add new Claims 160 through 163 (which find their support in the claims of the '307 application as originally filed) as follows:

--160 (new) A host bacterial cell comprising a DNA molecule, with or without a sequence modification within the polypeptide- encoding region thereof , said molecule encoding for a polypeptide having an amino acid sequence sufficiently duplicative of that of human CFTR to allow possession of the biological property of epithelial cell anion

channel regulation, wherein said DNA molecule is maintained in said cell such that the viability of said host bacterial cell is not substantially affected thereby.

161(new) A host bacterial cell according to Claim 160 wherein said DNA molecule is present therein in low copy number.

162(new) A host bacterial cell according to Claim 160 wherein said DNA molecule contains a point mutation within the encoding sequence thereof that substantially prevents recognition by said host bacterial cell of a cryptic bacterial promoter therein.

163(new) A host bacterial cell according to Claim 160 wherein the polypeptide-encoding region of said DNA molecule contains an intron that disrupts expression therefrom of toxic partial CFTR polypeptides.--

It will be noted that these claims do not present subject matter or limitations not already examined, but merely ensure that applicants gain the protection on host bacterial cells to which they are properly entitled.

Conclusion

No Request for an Extension of Time is believed to be necessary at this time since the Examiner's final action herein was mailed on June 16, 1994, and an extension fee covering the second month-after final was transmitted with Applicants' prior amendment herein on October 27, 1994. Additionally, **no fees for the additional claims** are believed to be due since the number of claims

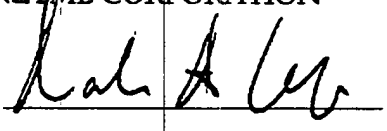
added is substantially outnumbered by the number of claims canceled herein by way of Applicants' paper of October 3, 1994. However, should the Patent Office determine that claim fees or extension fees are due, the Patent Office is hereby authorized to charge such fees to Deposit Account 07-1074. Pursuant to 37 CFR §§ 1.136(a), a Request for an Extension of Time is therefore requested hereinby.

Applicants believe that the application is in condition for allowance and it is respectfully requested that the claims now be passed to allowance. The Examiner is invited to phone either the undersigned at (508) 872-8400 x 2555, or Applicants counsel of record at LAHIVE and COCKFIELD, to discuss any matters that she believes require further attention. An early and favorable action is requested respectfully.

Respectfully submitted,

GENZYME CORPORATION

Dated: November 16, 1994

By: 

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Probing the basic defect in cystic fibrosis

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The concurrent developments in electrophysiology studies and the identification of the cystic fibrosis transmembrane conductance regulator (CFTR) gene has provided a unique opportunity to probe the basic cellular defect underlying cystic fibrosis. Various properties of the CFTR protein have been deduced from its primary sequence, the variety of mutations in patients and genotype–phenotype correlations, as well as the results of more recent DNA transfection studies. The most exciting observation is the fact that CFTR acts like a cAMP-regulated Cl^- channel.

Current Opinion in Genetics and Development 1991, 1:4–10

Introduction

Cystic fibrosis (CF) is a common autosomal recessive disorder prevalent in the Caucasian population (for a review see [1]). The gene responsible for this disease has recently been identified through molecular cloning studies based on the precise chromosome localization of the disease locus [2–4]. Currently, the most exciting challenge in CF research, as for any disease in which a genetic defect is found before a clear biochemical description of that defect is available, is to identify of the function or functions of the gene product. Of particular importance to CF, however, will be to understand how the same defect can cause problems in a variety of organs.

The abnormality in mucus secretion resulting in chronic obstructive lung disease and pancreatic enzyme insufficiency, together with the elevated levels of sweat electrolytes, are indicative of a general exocrine malfunction in CF [1]. A defect in the regulation of chloride transport has been the most consistent finding in studies of CF epithelial cells (Fig. 1; reviewed in [5•,6•]). This defect appears to be characterized by the lack of a cAMP-mediated channel response, but there have been different descriptions regarding the properties of the chloride channel involved [7–13]. Early single channel patch-clamp studies suggested that the channel is outwardly rectifying with a conductivity of 26–50 picoSiemens [7,8], whereas the more recent measurements indicate that it may be a small linear channel of 4–8 picoSiemens [11–13].

The concurrent developments in CF electrophysiology studies and the identification of the gene has provided a unique opportunity to probe the basic cellular defect underlying this disease. In the past 18 months there has been significant progress in understanding the relationship between the observed defect in chloride transport and the putative protein product of this gene, named the cystic fibrosis transmembrane conductance regulator (CFTR).

Predicted properties of the encoded protein

From its cDNA sequence, CFTR is predicted to contain 1480 amino acid residues, and to have a relative molecular mass of about 170 000 and a pI of 8–9 [3]. In addition, the protein appears to be internally duplicated: there are two repeated motifs, each consisting of six putative membrane-spanning regions followed by an ATP-binding site denoted as the nucleotide-binding fold (NBF) (Fig. 2). The hydrophilic segment preceding the first transmembrane sequence is probably in the cytoplasm, and the bulk of the protein is presumably also internally located. The largest predicted extracellular loop is between transmembrane segments 7 and 8, and contains two possible N-linked glycosylation sites. These features are reminiscent of those observed for many other membrane-associated transport proteins in prokaryotes and eukaryotes [3,14•].

The R-domain is a highly charged cytoplasmic region linking the protein halves (Fig. 3). This unique segment is not found in related transport proteins, and the domain is operationally defined by a single large exon that encodes this sequence. Of the 241 amino acids encoded in this exon, 69 are polar residues, arranged in alternating clusters of positive and negative charges. In addition, nine of the ten potential sites for phosphorylation by protein kinase A and seven of the putative protein kinase C phosphorylation targets found in CFTR are located in this domain. Thus, the R-domain is thought to play an important role in CFTR function [3].

The presence of ATP-binding site consensus sequences (the Walker A and B motifs) in CFTR suggests that ATP

Abbreviations

CF—cystic fibrosis; CFTR—cystic fibrosis transmembrane conductance regulator; NBF—nucleotide-binding fold; PI—pancreatic insufficient; PS—pancreatic sufficient.

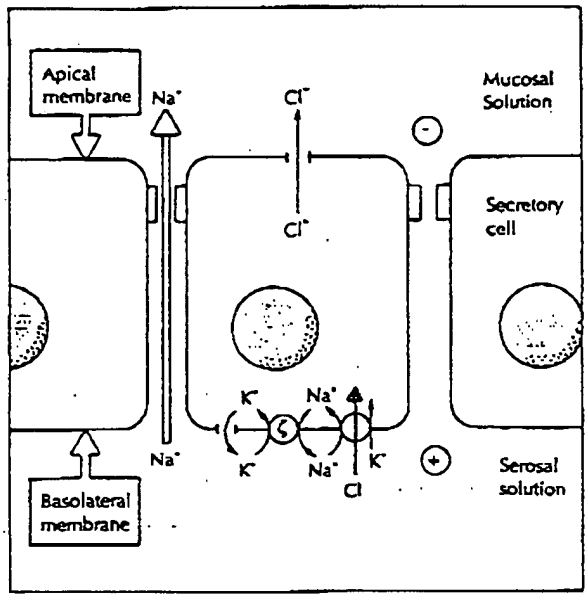

Fig. 1. Cellular model for trans-epithelial chloride secretion (adapted from [38]). Cl^- enters secretory cells across the basolateral membrane, coupled to the entry of Na^+ and K^+ via a loop diuretic-sensitive Na^+–K^+–$2Cl^-$ cotransport process. Sodium is returned to the serosal (plasma-facing) solution by the ouabain-sensitive Na^+–K^+ pump, which also brings K^+ into the cell. Cellular K^+ is returned to the serosal solution via basolateral K^+ channels. These three processes lead to cellular Cl^- accumulation at levels exceeding the electrochemical potential of extracellular Cl^-. Cl^- then exits across the apical membrane by diffusion through Cl^--sensitive channels. Na^+ accompanies Cl^- to the luminal solution by diffusing across the paracellular pathway, driven by the trans-epithelial voltage arising from electrogenic Cl^- transport. The defect in CF cells is thought to be at the point of regulation of Cl^--sensitive channels.

binding (and possibly its hydrolysis) is necessary for the function of the protein [3,14•]. The first experimental evidence for the involvement of ATP in CFTR function came from a study by Thomas *et al.* [15•], who demonstrated binding of ATP to a 67-residue synthetic peptide spanning the central region of the first NBF. This peptide contains the Walker A motif and the central region of the consensus ATP-binding site, but not the B motif; nevertheless, this does not exclude involvement of the B motif in the native protein.

Based on the primary sequence and its structural resemblance to other transport proteins, it is intuitive to suggest that CFTR is an epithelial cell-specific transporter. For example, Hyde *et al.* [14•] argue the following: channels, unlike transporters, do not require ATP hydrolysis, whereas CFTR has two NBFs; ion flow through channels is bidirectional, whereas CFTR resembles a family of unidirectional transporters; a Cl^- channel is expected to have a turnover rate far more rapid than that of transporters; Cl^- channels appear to have varying properties in different tissues, whereas CF affects a variety of tissues; and finally, the molecular weight of a putative Cl^- channel is much less than that of CFTR. Ringe and Pestko [16] further speculate that the products of the arachidonic acid pathway, leukotrienes and prostaglandins, are possible substrates for CFTR. Although these are attractive hypotheses, a different conclusion has been derived from cDNA transfection studies (see below).

Identification of naturally occurring mutations

Useful information about the structure and function of CFTR may be derived from the characterization of both naturally occurring mutations and normal sequence variants. An international consortium (the CF Genetic Analysis Consortium), which consists of 86 research groups from more than 20 countries, has been established to collect this information. To date, over 75 different disease-causing mutations have been reported.

The most common CF mutation (ΔPhe508, or ΔF508 in one-letter code), which accounts for 68% of the global defective gene pool [4,17], is a 3 bp deletion located in exon 10. The primary consequence of this mutation is the deletion of a single phenylalanine residue at position 508, which lies within the first NBF of the predicted polypeptide between the consensus Walker A and B motifs. It has been suggested that ΔPhe508 may prevent proper binding or hydrolysis of ATP, or cause a conformational change affecting CFTR activity [3].

The structural and functional importance of the region surrounding Phe508 has been further elucidated by several additional observations. First, a second 3 bp deletion has been found in the vicinity of ΔPhe508 [18]. This mutation, which is the only other single amino acid deletion found in CFTR so far, deletes the isoleucine residue at position 506 or 507 (hence denoted as ΔIle507). Second, amino acid substitutions for Phe508 and Ile506 have been found in two rare variant alleles that are apparently normal (with either a cysteine residue at position 508 or a valine residue at 506) [19]. Third, direct analysis of the physical properties of a synthetic peptide containing these amino acid residues suggests that deletion of any of the residues could induce a significant structural change in the predicted β-sheet structure and also affect ATP binding [15•]. These observations are consistent, therefore, with the hypothesis that the length of the polypeptide is more important than the identity of the actual amino acid residues in this region [18].

A number of missense mutations have been found within the two NBFs, some of them affecting the highly conserved residues in the Walker motifs (e.g. Gly458 [20], Ser549 [18], and Gly551 [18,21]. These mutations thus confirm that ATP binding (and possibly hydrolysis) is essential for CFTR function. Those mutations that affect non-conserved amino acids (e.g. Asp110→His, Arg117→His, Arg347→Pro, Ala559→Thr, Tyr563→Asn, and Pro574→His [18,21,22]) are perhaps more revealing because the importance of these residues could not otherwise be easily recognized. Understanding the role of these missense mutations may be more difficult at the present time, however, as the three-dimensional structure of the protein is unknown.

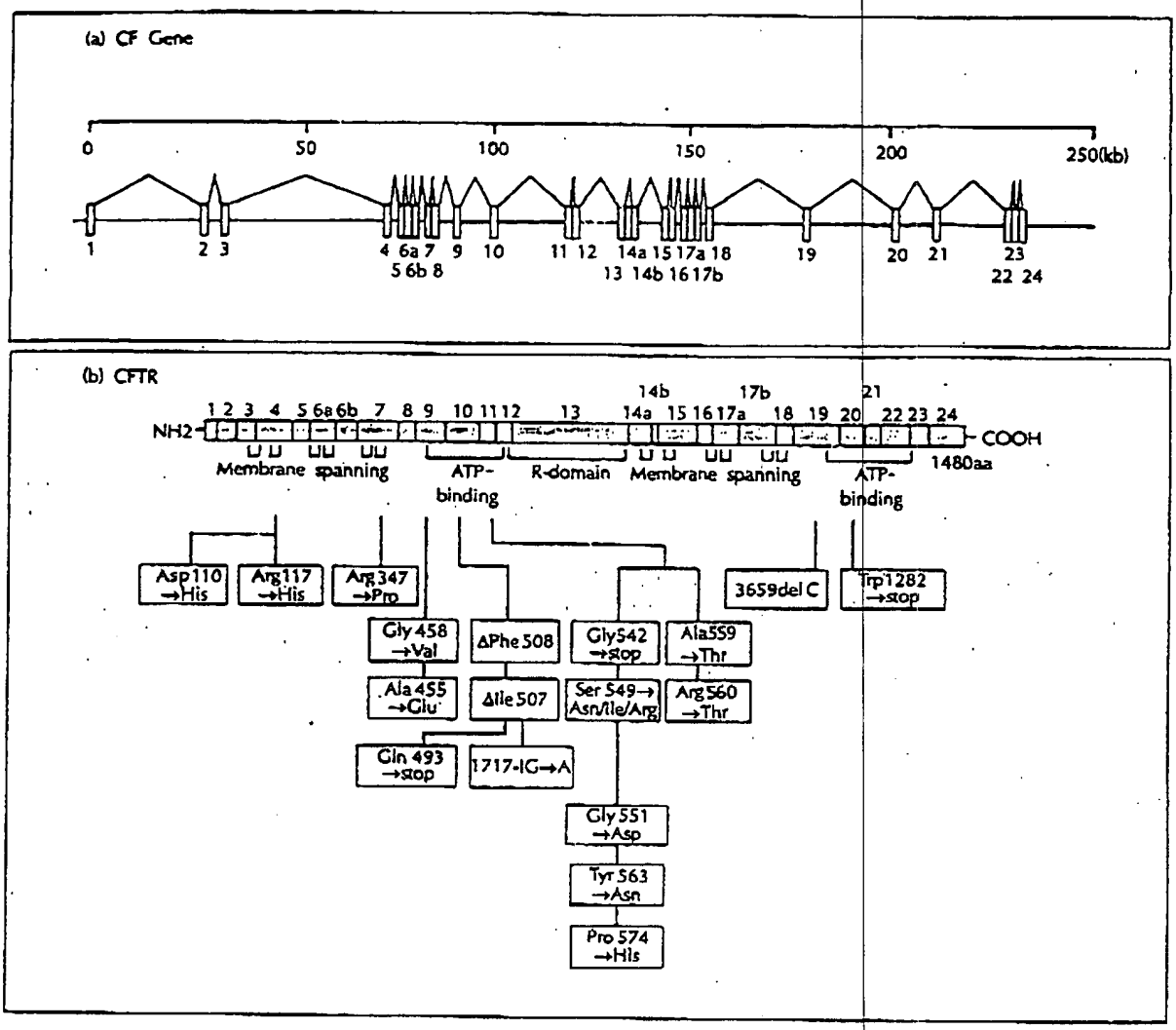

Fig. 2. Schematic diagram (adapted from [39]) of the exon–intron organization of the CFTR gene (a), and the putative domain-type structure of its product (b). The gene is composed of 27 exons. The nomenclature 6a, 6b, 14a, 14b, 17a and 17b reflects intron sequences not originally reported [2]. The positions of the CF mutations, including the major mutation ΔPhe508, are indicated.

The mutation screening data also confirm that the predicted initiation codon [3] is correct; thus, in addition to Asp110→His and Arg117→His [22], there are more than ten other mutations known to precede the next available methionine codon, located in exon 4 (amino acid position 150), indicating that the sequence between exons 1 and 4 is essential for CFTR function.

Approximately half of the known CF mutations are nonsense, frameshift or mRNA splicing mutations (unpublished data reported to the CF Genetic Analysis consortium) [18,20–25]. Although mechanisms such as alternative splicing possibly prevent premature termination of CFTR synthesis for some of these mutant alleles, it is more probable that most, if not all, will produce a null phenotype. Although most of these mutations are detected in compound heterozygotes because of their relatively low individual frequency, there are some examples of CF patients homozygous for such CF mutations who are thought to have a null CFTR phenotype [20,23]. These observations suggest that total absence of CFTR is not lethal and, moreover, patients with a presumed null CFTR phenotype can have merely a mild clinical course of CF [20,23].

Despite extensive searches, only two frameshift mutations have been found towards the end of the R-domain [24,25]. In addition, no sequence polymorphism has been noted in this region among the large number of normal and CF alleles examined (the CF Genetic Analysis Consortium) [18]. The significance of these observations is unknown, but they are consistent with the assumption that the R-domain is essential for CFTR function.

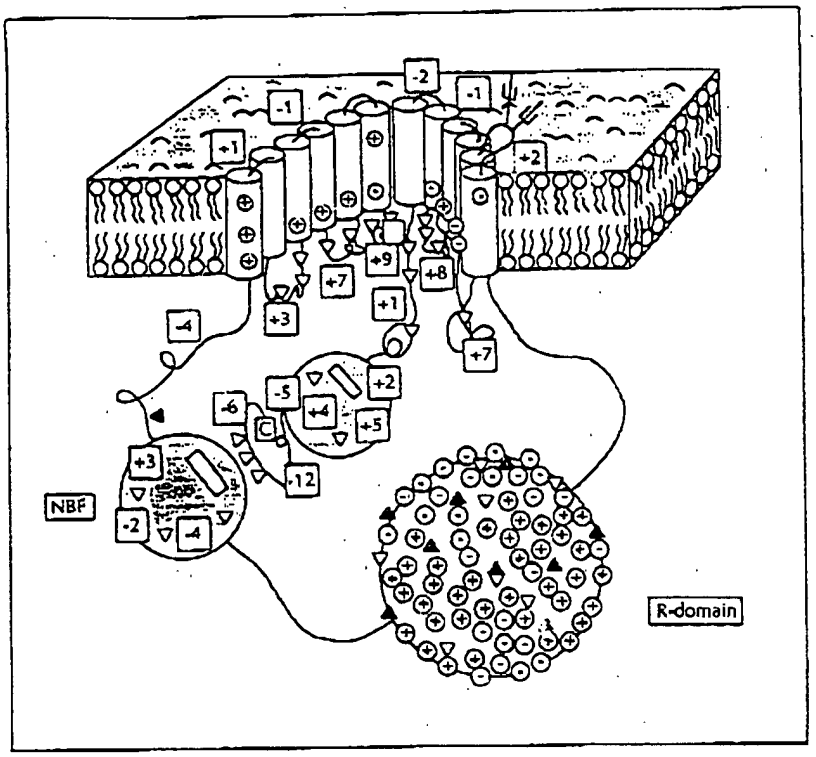

Fig. 3. Schematic model of CFTR (adapted from [3]). The six predicted membrane-spanning helices in each half of the molecule are depicted as cylinders. The position at which ATP enters each of the cytoplasmically-oriented nucleotide-binding folds (NBFs) is marked. The large polar R-domain, which links the two halves, is represented by a sphere. Individual charged amino acid residues are shown as small circles containing the appropriate charge sign. Net charges on the internal and external loops joining the membrane cylinders and on regions of the NBFs are written in boxes. Potential sites for phosphorylation by protein kinase A (▲) or C (▽) and N-glycosylation (ψ) are indicated. The symbol ⊕ denotes the amino acid residue Lys, Arg or His, and the symbol ⊖ denotes the amino acid residue Asp or Glu.

Genotype–phenotype correlation

The remarkable concordance of pancreatic involvement among CF patients of the same family has provided the first suggestion that mutations in the CF gene have a direct clinical consequence [26]. Kerem and coworkers [4] have proposed that there are 'severe' and 'mild' mutations that determine the level of pancreatic function. Approximately 90% of CF chromosomes are believed to carry a severe allele, such as ΔPhe508, whereas the rest carry a mild allele. About 85% of patients, who are pancreatic insufficient (PI), are thought to have two severe alleles; the other 15% of CF patients, who are pancreatic sufficient (PS), are thought to have at least one mild allele. Not all homozygous ΔPhe508 patients are diagnosed as being PI, however. These exceptions may constitute the 10–20% of PS patients whose pancreatic function deteriorates at later stage [27].

Besides ΔPhe508, at least 10 other mutations (Glu493→stop, Δlle507, 1717-1G→1A, Gly542→stop, Ser549→Ile, Ser549→Arg, Gly551→Asp, Arg560→Thr, 3659delC and Trp1282→stop) are classified as severe alleles [18,20,28]. Only three mild alleles (Ala455→Glu, Tyr563→Asn and Pro574→His) have been defined so far [18]. It is of interest to note that the severe alleles are generally not expected to produce complete CFTR, whereas the mild alleles are missense mutations.

Although most PS patients tend to have better lung function than PI patients, there does not seem to be a direct correlation between genotype and pulmonary phenotype [29•]. Thus, additional genetic and environmental factors are clearly involved in determining lung function, and it is also possible that the role of CFTR is different in different organs. A better understanding of the phenotypes awaits a more detailed analysis of the function of CFTR itself.

Construction of a full-length CFTR cDNA

The cloning of the CFTR gene offers the potential to produce the protein in various expression systems for further biochemical and physiological analyses. Because of the large size of the gene (250 kb) and the lack of knowledge about its promoter region, most studies have used a full-length cDNA insert expressed under the control of a heterologous promoter. The reconstruction of a full-length cDNA from the previously isolated overlapping fragments [3] has been hampered, however, by the instability of a DNA sequence within the coding region (JM Rommens, S Dho, CE Bea, N Kartner, D Kennedy, JR Riordan, L-C Tsui and JK Foshett, unpublished data) [31••,32••]. This technical difficulty was eventually overcome by the use of a low-copy number bacterial plasmid [30••] and, independently, by introducing nucleotide substitutions within the presumptive unstable region [31••–33••]. Such constructs were used for most of the transfection experiments discussed below.

Detection of CFTR protein

A set of specific antibodies directed against CFTR would be extremely valuable in the study of this protein. Unfortunately, cross-reactive materials of unknown nature are also detected by many of the antibodies directed against synthetic peptides or fusion proteins, even in cells that do not appear to express CFTR (on the basis of RNA detection). Nevertheless, with proper controls, and through the use of heterologous transfection systems, Gregory *et al.* [30••] and Kartner *et al.* [32••] have shown that mature CFTR corresponds to a diffuse band of 150–170 kD on SDS–polyacrylamide gels. The proteins synthesized using full-length CFTR transcripts *in vitro* and in expression systems lacking glycosylation activity have a much faster mobility (130–140 kD) [30••,32••]. The difference in mobility is, therefore, probably a result of glycosylation in the mature protein, although the apparent molecular weight of the unglycosylated form is much lower than that predicted from the amino acid sequence. The cause of this aberrant mobility is unknown, but the observation appears to be common among membrane-associated proteins.

The problem of glycosylation has also been studied by Cheng *et al.* [33••] by expressing a number of mutant CFTR variations in monkey COS-7 cells. These transfection studies show that glycosylation is incomplete for a number of mutant proteins (e.g. ΔPhe508, ΔIle507, Lys464→Met, Phe508→Arg and Ser549→Ile). It is suggested that these mutant proteins are recognized as abnormal and thus retained in the endoplasmic reticulum, where they are subsequently degraded. These investigators further propose that the molecular basis of most CF is the absence of mature CFTR at the correct cellular location [33••]. This model, although appealingly straightforward, cannot account for those mutants in which glycosylation appears to be normal (e.g. Arg334→Trp, Gly551→Asp and Lys1250→Met). It may be the case that mutant proteins do predispose slightly different clinical phenotypes depending on whether glycosylation is normal or not, but all the mutants mentioned above appear to be in the severe class, irrespective of glycosylation status. Moreover, it is unclear if glycosylation is required for function [32••]. Therefore, the value of the COS cell transfection system needs further consideration.

Detection of cAMP-regulated chloride conductance

Two independent studies have recently demonstrated that introduction of a CFTR cDNA clone into cells derived from CF patients can confer cAMP-inducible Cl^- permeability like that present in normal cells and absent in CF cells [31••,34••].

In one study, Rich *et al.* [34••] introduced a CFTR-expressing clone into primary and transformed CF airway epithelial cells, using the vaccinia virus expression system. Cl^- channel activity, as measured by iodide flux, was monitored in a single cell assay system in which the transfected cells were loaded with the halide-sensitive dye SPQ (6-methyl-N-(3-sulfopropyl)quinolinium). Upon induction of intracellular cAMP levels with forskolin, increase of I^- flux was more rapid in cells transfected with the normal cDNA than in those transfected with the variant containing the ΔPhe508 mutation. There was considerable cell to cell variability in the response, however. Nevertheless, patch clamp measurements show that cAMP-induced whole cell current were significantly higher in cells with the normal CFTR than in those with the ΔPhe508 variant.

In the other study, Drumm *et al.* [31••] used the retrovirus-mediated gene transfer method to introduce a CFTR-expressing clone into a CF pancreatic adenocarcinoma cell line, CF-PAC. Although clonal variation was also observed, a significant increase of ^{125}I efflux in the cells containing intact CFTR was observed upon forskolin treatment. In addition, a substantial increase in whole cell current was observed only in cells expressing intact CFTR, whereas no increase in current was detectable in cells containing the control vector. The current–voltage relationship measured for the activated channel is indicative of a linear channel with selectivity for anions.

Although studies described above serve to confirm the identity of the gene encoding CFTR and to establish the necessary first step in developing gene therapy strategies for treating the disease, they provide no further information about the function of the protein. It is unclear from these experiments whether the appearance of cAMP-regulated Cl^- conductance in the CF epithelial cells is a result of the restoration of regulation of existing channels, or the introduction of new regulatable channels. An important insight into this question was provided, however, by a series of transfection studies with non-epithelial cell types (JM Rommens, S Dho, CE Bear, N Kartner, D Kennedy, JR Riordan, L-C Tsui and JK Foskett, unpublished data) [32••,35••].

Using the vaccinia virus system, Anderson *et al.* [35••] transfected the CFTR gene into HeLa, Chinese hamster ovary and NIH 3T3 fibroblast cells, and detected an increased anion permeability and chloride current in the presence of cAMP. Similar increases were not observed for the non-transfected control or for cells expressing a mutant CFTR (ΔPhe508). The cAMP-activated Cl^- currents were comparable to those seen in epithelial cells expressing endogenous CFTR or CF cells expressing exogenous CFTR. Comparable observations were made by Kartner *et al.* [32••] for insect cells (Sf9) transiently transfected with a baculovirus construct containing a full length CFTR cDNA insert. The current–voltage relationship for the cAMP-induced current also appears to be linear, and the single channel conductance is low (8 picoSiemens). We have demonstrated that it is possible to establish stable mouse fibroblast (L) cell lines expressing CFTR and to detect the same cAMP-regulated Cl^- channel activity. Furthermore, the levels of CFTR expression and activity in the transfected L cells are highly reproducible, and are comparable to those observed in human epithelial cell lines.

Results from the transfection studies in non-epithelial cells strongly suggest, therefore, that CFTR itself is a regulated Cl^- channel. Although the formal possibility that CFTR merely serves to regulate latent Cl^- channels in the transfected cells cannot be completely excluded, the presence of channels with the same properties in different cells types from different species argues against this interpretation. On the other hand, it remains to be explained how CFTR acts as a Cl^- channel, especially as the primary structure of CFTR is more suggestive of an active transporter than a channel. Can the R-domain perform the necessary magic? In addition, the current–voltage relationship measured for the CFTR channel is apparently linear, whereas the previous single channel data showed that the defective regulation observed in CF cells affected an outwardly rectifying Cl^- channel. It is also necessary to explain a number of other abnormalities observed in CF cells, such as in Na^+ transport [36] and sulfation [37].

Perspective

It is possible that most of the observed defects in CF cells are a result of secondary consequences. A compromise suggestion is that CFTR is a multifunctional protein, acting as a channel as well as being capable of regulating other cellular activities. Judging by the speed of research in CF nowadays, answers to these questions will probably reach the press before this article leaves the editor's desk.

References and recommended reading

Papers of special interest, published within the annual period of review, have been highlighted as:

- • of interest
- •• of outstanding interest

1. BOAT TF, WELSH MJ, BEAUDET AL: Cystic Fibrosis. In *The Metabolic Basis of Inherited Disease (6th edn)* edited by Scriver CR, Beaudet AL, Sly WS, Valle D [book]. New York: McGraw-Hill, 1989, pp. 2649–2680.

2. ROMMENS JM, IANNUZZI MC, KEREM B, DRUMM ML, MELMER G, DEAN M, ROZMAHEL R, COLE JL, KENNEDY D, HIDAKA N, *ET AL*: Identification of the Cystic Fibrosis Gene: Chromosome Walking and Jumping. *Science* 1989, 245:1059–1065.

3. RIORDAN JR, ROMMENS JM, KEREM B, ALON N, ROZMAHEL R, GRZELCZAK Z, ZIELENSKI J, LOK S, PLAVSIC N, CHOU J-L, *ET AL*: Identification of the Cystic Fibrosis Gene: Cloning and Characterization of Complementary DNA. *Science* 1989, 245:1066–1073.

4. KEREM B, ROMMENS JM, BUCHANAN JA, MARKIEWICZ D, COX TK, CHAKRAVARTI A, BUCHWALD M, TSUI L-C: Identification of the Cystic Fibrosis Gene: Genetic Analysis. *Science* 1989, 245:1073–1080.

5. • QUINTON PM: Cystic Fibrosis: a Disease in Electrolyte Transport. *FASEB J* 1990, 4:2709–2717.
A review of electrolyte transport in CF patients.

6. • WELSH MJ: Abnormal Regulation of Ion Channels in Cystic Fibrosis Epithelia. *FASEB J* 1990, 4:2718–2725.
A review of the electrophysiology of CF epithelial cells.

7. WELSH MJ, LIEDKE CM: Chloride and Potassium Channels in Cystic Fibrosis Airway Epithelia. *Nature* 1986, 322:467–470.

8. FRIZZELL RA, RECHKEMMER G, SHOEMAKER RL: Altered Regulation of Airway Epithelial Cell Chloride Channels in Cystic Fibrosis. *Science* 1986, 233:558–560.

9. HWANG T-C, LI L, ZEITLIN PL, GRUENERT DC, HUGANIR AR, GUGGINO WB: Cl^- Channels in CF: Lack of Activation by Protein Kinase C and cAMP-Dependent Protein Kinase. *Science* 1989, 244:1351–1353.

10. LI M, MCCANN JD, ANDERSON MP, CLANCY JP, LIEDKE CM, NAIRN AC, GREENGARD P, WELSH MJ: Regulation of Chloride Channels by Protein Kinase C in Normal and Cystic Fibrosis Airway Epithelia. *Science* 1989, 244:1353–1356.

11. GRAY MA, HARRIS A, COLEMAN L, GREENWELL JR, ARGENT BE: Two Types of Chloride Channels on Duct Cells Cultured from Human Fetal Pancreas. *Am J Physiol* 1989, 257:C240–C251.

12. CLIFF WH, FRIZZELL RA: Separate Cl^- Conductances Activated by cAMP and Ca^{2+} in Cl^- Secreting Epithelial Cells. *Proc Natl Acad Sci USA* 1990, 87:4956–4960.

13. TABCHARANI JA, LOW W, ELIE D, HANRAHAN JW: Low-Conductance Chloride Channel Activated by cAMP in the Epithelial Cell Line T84. *FEBS Letters* 1990, 270:157–164.

14. • HYDE SC, EMSLEY P, HARTSHORN MJ, MIMMACK MM, GILEADI U, PEARCE SR, GALLAGHER MP, GILL DR, HUBBARD RE, HIGGINS CF: Structural Model of ATP-binding Proteins Associated with Cystic Fibrosis, Multidrug Resistance and Bacterial Transport. *Nature* 1990, 346:362–365.
A tertiary structure model of the ATP-binding domain of a class of transport proteins is presented. The modeling is based on similarities between the predicted secondary structures of members of this family and the previously determined structure of adenylate kinase. The authors include CFTR in this class of proteins, and argue that CFTR is probably not a chloride channel.

15. • THOMAS PJ, SHENBAGAMURTHI P, YSERN X, PEDERSEN PL: Cystic Fibrosis Transmembrane Conductance Regulator: Nucleotide Binding to a Synthetic Peptide. *Science* 1991, 251:555–557.
A peptide 67 amino acids in length, corresponding to the central region of the nucleotide-binding fold of CFTR, has been chemically synthesized and purified. This peptide is capable of binding adenine nucleotides, with the binding of ATP having the highest affinity. Circular dichroism spectroscopy shows that this peptide assumes a predominantly β-sheet structure, conformation of which can be significantly altered by deletion of Phe508.

16. RINGE D, PETSKO GA: Cystic Fibrosis: A Transport Problem? *Nature* 1990, 346:312–313.

17. THE CYSTIC FIBROSIS GENETIC ANALYSIS CONSORTIUM: Worldwide Survey of the ΔF508 Mutation — Report from the Cystic Fibrosis Genetic Analysis Consortium. *Am J Hum Genet* 1990, 47:354–359.

18. KEREM B, ZIELENSKI J, MARKIEWICZ D, BOZON D, GAZIT E, YAHAV J, KENNEDY D, RIORDAN JR, COLLINS FS, ROMMENS JR, TSUI L-C: Identification of Mutations in Regions Corresponding to the Two Putative Nucleotide (ATP)-Binding Folds of the Cystic Fibrosis Gene. *Proc Natl Acad Sci USA* 1990, 87:8447–8451.

19. KOBAYASHI K, KNOWLES M, O'BRIEN WE, BEAUDET AL: Benign Missense Variations in the Cystic Fibrosis Gene. *Am J Hum Genet* 1990, 47:611–615.

20. CUPPENS H, MARYNEN P, DE BOECK C, CASSIMAN JJ: Study of the G542X and G458V Mutations in a Sample of Belgian Patients. *Pediatr Pulmonol* 1990, 5 (Suppl):205.

21. CUTTING GR, KASCH LM, ROSENSTEIN BJ, ZIELENSKI J, TSUI L-C, ANTONARAKIS SE, KAZAZIAN HH JR: A Cluster of Cystic Fibrosis Mutations in the First Nucleotide-Binding Fold of the Cys-

tic Fibrosis Conductance Regulator Protein. *Nature* 1990, 346:366–369.

22. DEAN M, WHITE M, AMOS J, GERRARD B, STEWART C, KHAW K-T, LEPPERT M: Multiple Mutations in Highly Conserved Residues are Found in Mildly Affected Cystic Fibrosis Patients. *Cell* 1990, 61:863–870.

23. CUTTING GR, KASCH LM, ROSENSTEIN BJ, TSUI L-C, KAZAZIAN HH JR, ANTONARAKIS SE: Two Patients with Cystic Fibrosis, Nonsense Mutations in Each Cystic Fibrosis Gene, and Mild Pulmonary Disease. *New Eng J Med* 1990, 323:1685–1689.

24. ZIELENSKI J, BOZON D, KEREM B, MARKIEWICZ D, DURIE P, ROMMENS JM, TSUI L-C: Identification of Mutations in Exons 1 Through 8 of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Gene. *Genomics* 1991, 10, in press.

25. WHITE MB, AMOS J, HSU JMC, GERRARD B, FINN P, DEAN M: A Frame-Shift Mutation in the Cystic Fibrosis Gene. *Nature* 1990, 344:665–667.

26. COREY M, DURIE P, MOORE D, FORSTNER GG, LEVISON H: Familial Concordance of Pancreatic Function in Cystic Fibrosis. *J Pediatr* 1989, 115:274–279.

27. WATERS DL, DORNEY SFA, GASKIN KJ, GRUCA MA, O'GHALLORAN M, WILCKEN B: Pancreatic Function in Infants Identified as Having Cystic Fibrosis in a Neonatal Screening Program. *New Engl J Med* 1990, 322:303–308.

28. VIDAUD M, FANEN P, MARTIN J, GHANEM N, NICOLAS S, GOOSSENS M: Three Mutations in the CFTR Gene in French Cystic Fibrosis Patients: Identification by Denaturing Gradient Gel Electrophoresis. *Hum Genet* 1990, 85:446–449.

29. • KEREM E, COREY M, KEREM B, ROMMENS J, MARKIEWICZ D, LEVISON H, TSUI L-C, DURIE P: Association Between the ΔF508 Mutation and Phenotypes in Cystic Fibrosis. *New Engl J Med* 1990, 323:1517–1522.

This paper describes a correlation study between the genotypes and phenotypes of 293 CF patients. The data suggest that the variable clinical course in CF patients can be attributed to specific genotypes at the CF locus.

30. •• GREGORY RJ, CHENG SH, RICH DR, MARSHALL J, PAUL S, HEHIR K, OSTEDGAARD L, KLINGER KW, WELSH MJ, SMITH AE: Expression and Characterization of the Cystic Fibrosis Transmembrane Conductance Regulator. *Nature* 1990, 347:382–386.

CFTR synthesis is monitored in a reticulocyte translation system and in a transfection assay based on the vaccinia virus–T7 polymerase hybrid expression system. These studies show that CFTR normally exists in a glycosylated form in mammalian cells.

31. •• DRUMM ML, POPE HA, CLIFF WH, ROMMENS JM, MARVIN SA, TSUI L-C, COLLINS FS, FRIZZELL RA, WILSON JM: Correction of the Cystic Fibrosis Defect *In Vitro* by Retrovirus-Mediated Gene Transfer. *Cell* 1990, 62:1227–1233.

The retrovirus-mediated gene transfer procedure is used to introduce the full length CFTR cDNA into a pancreatic adenocarcinoma cell line derived from a CF patient. This cell line shows the Cl^- transport abnormalities characteristic of CF but, upon acquisition of the CFTR cDNA, a cAMP-inducible chloride permeability can be detected. This result indicates that expression of the normal CFTR gene confers cAMP-dependent chloride channel regulation on CF epithelial cells.

32. •• KARTNER N, HANRAHAN JW, JENSEN TJ, NAISMITH AL, SUN S, ACKERLEY CA, REYES EF, TSUI L-C, ROMMENS JR, BEAR CE, RIORDAN JR: Expression of the Cystic Fibrosis Gene in Non-Epithelial Invertebrate Cells Produces a Regulated Anion Conductance. *Cell* 1991, 64:681–691.

The baculovirus expression system is used to express CFTR in Sf9 insect cells. Concomitant with CFTR expression, these cells exhibit a new cAMP-stimulated anion permeability. The conductance resembles that present in several CFTR expressing human epithelial cells. This finding demonstrates that CFTR can function in heterologous non-epithelial cells, and lends support to the possibility that CFTR may itself be a regulated anion channel.

33. •• CHENG SH, GREGORY RJ, MARSHALL J, PAUL S, SOUZA DW, WHITE GA, O'RIORDAN CR, SMITH AE: Defective Intracellular Transport and Processing of CFTR is the Molecular Basis of Most Cystic Fibrosis. *Cell* 1990, 63:827–834.

Mutant CFTR constructs are introduced into monkey COS-7 cells. The study shows that mature, fully glycosylated CFTR is absent from cells containing a subset of the mutant constructs. Instead, an incompletely glycosylated version of the protein is detected. The authors propose that the mutant proteins are recognized as abnormal and remain incompletely processed in the endoplasmic reticulum, where they are subsequently degraded. The glycosylation sites previously predicted [3] appear to be used.

34. •• RICH DR, ANDERSON MP, GREGORY RJ, CHENG SH, PAUL S, JEFFERSON DM, MCCANN JDM, KLINGER KW, SMITH AE, WELSH MJ: Expression of Cystic Fibrosis Transmembrane Conductance Regulator Corrects Defective Chloride Channel Regulation in Cystic Fibrosis Airway Epithelial Cells. *Nature* 1990, 347:358–363.

The vaccinia virus expression system is used to introduce the full-length CFTR cDNA into primary and transformed respiratory cells derived from CF patients. Iodide flux and patch-clamp measurements show that cells transfected with a normal CFTR but not the ΔPhe508 cDNA have a more rapid iodide flux in the presence of forskolin. The results are taken to mean that expression of CFTR corrects the defective Cl^- channel regulation in these cells.

35. •• ANDERSON MP, RICH DP, GREGORY RJ, SMITH AE, WELSH MJ: Generation of cAMP-Activated Chloride Currents by Expression of CFTR. *Science* 1991, 251:679–682.

The CFTR gene was introduced into HeLa, Chinese hamster ovary and NIH 3T3 mouse fibroblast cells using the vaccinia virus transfection and expression system. Increases in anion permeability and chloride current were detected in CFTR-expressing cells treated with cAMP, but not in cells expressing a mutant protein or in non-transfected cells. The simplest interpretation of these observations is that CFTR is itself a cAMP-activated chloride channel.

36. BOUCHER RC, STUTTS MJ, KNOWLES MR, CANTLEY L, GATZY JT: Na^+ Transport in Cystic Fibrosis Respiratory Epithelia. Abnormal Basal Rate and Response to Adenylate Cyclase Activation. *J Clin Invest* 1986, 78:1245–1252.

37. CHENG P-W, BOAT TF, CRANFILL K, YANKASKAS JR, BOUCHER RC: Increased Sulfation of Glycoconjugates by Cultured Nasal Epithelial Cells from Patients with Cystic Fibrosis. *J Clin Invest* 1989, 84:68–72.

38. FRIZZELL RA, HALM DR: Chloride Channel in Epithelial Cells. *Curr Top Membr Transport* 1990, 37:247–282.

39. ZIELENSKI J, ROZMAHEL R, BOZON D, KEREM B, GRZELCZAK Z, RIORDAN JR, ROMMENS JM, TSUI L-C: Genomic DNA Sequence of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Gene. *Genomics* 1991, 10, in press.

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